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UP-REGULATION OF RENAL Na^+, K^+-ATPase: THE POSSIBLE NOVEL MECHANISM OF LEPTIN-INDUCED HYPERTENSION

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Hyperleptinemia may be involved in the pathogenesis of obesity-associated hypertension, however, the mechanism of hypertensive effect of leptin has not been elucidated. We investigated the effect of experimental hyperleptinemia on renal function, renal Na^+, K^+-ATPase and ouabain-sensitive H^+, K^+-ATPase activities in the rat. Leptin administered for 7 days (0.25 mg/kg twice daily *sc*) decreased food intake on 6th and 7th day of treatment but had no effect on body weight. Systolic blood pressure was 30.5% higher in leptin-treated animals. Urinary excretion of sodium decreased by 35.0% following leptin treatment. Leptin had no effect on potassium and phosphate excretion as well as on creatinine clearance. The activity of Na^+, K^+-ATPase in the renal cortex and medulla was higher in leptin-treated rats by 32.4% and 84.2%, respectively. In contrast, leptin had no effect on either cortical or medullary ouabain-sensitive H^+, K^+-ATPase. In pair-fed group, in which food intake was reduced to the level observed in leptin-treated group, no changes in sodium metabolism and renal Na^+, K^+-ATPase were observed. Leptin decreased urinary excretion of nitric oxide metabolites by 55.0% and urinary excretion of cGMP by 26.3%. Plasma concentration of atrial natriuretic peptide tended to be higher and urinary excretion of urodilatin was 64.9% higher in leptin-treated animals. These data suggest that hyperleptinemia decreases natriuresis by up-regulating Na^+, K^+-ATPase and stimulating tubular sodium reabsorption. This effect is mediated, at least in part, by deficiency of nitric oxide (NO). Abnormal renal sodium retention and vasoconstriction associated with NO deficiency may contribute to leptin-induced hypertension and to blood pressure elevation in hypertensive obese individuals.

Key words: *leptin, arterial hypertension, natriuresis, Na^+, K^+-ATPase, nitric oxide, atrial natriuretic peptide*

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INTRODUCTION

Arterial hypertension is one of the most important complications of obesity, leading to the development of atherosclerosis, ischemic heart disease, heart failure, and finally to increased mortality among obese individuals. The pathogenesis of obesity-associated hypertension is complex and incompletely understood [21]. Leptin, secreted by white adipose tissue, is primarily involved in the regulation of food intake and energy expenditure [45]. However, leptin receptors are found in many tissues including cardiovascular system and the kidney [31]. Obese individuals are characterized by hyperleptinemia, reflecting overproduction of the hormone by adipocytes and resistance of hypothalamic satiety centre to its anorectic effect [13]. Recent studies suggest the important role of leptin in obesity-related cardiovascular diseases including arterial hypertension. Chronic leptin administration increases blood pressure in experimental animals [37]. Moreover, hypertension is observed in transgenic mice overexpressing leptin, although their body weight is lower than in wild-type littermates [2]. In addition, obesity is accompanied by hypertension in hyperleptinemic agouti yellow obese mice but not in leptin-deficient ob/ob mice [32]. Some studies indicate that plasma leptin concentration correlates with blood pressure in hypertensive humans, also in those who are not obese [1]. The mechanism of hypertensive effect of leptin is not clear. It has been suggested that leptin increases blood pressure by stimulating sympathetic nervous system [2, 14, 17, 23], however, this mechanism cannot entirely explain the hypertensive effect of the adipose tissue hormone [10].

Abnormal renal sodium metabolism plays an important role in obesity-associated hypertension [20, 22], but the involvement of leptin in this process has not been investigated. In the present study, we examined the effect of experimental hyperleptinemia on electrolyte balance and the activity of renal Na^+, K^+-ATPase which drives active tubular sodium transport. In addition, we measured the activity of a related enzyme, ouabain-sensitive H^+, K^+-ATPase, involved in potassium reabsorption and urine acidification. Finally, we investigated the possible mechanisms through which hyperleptinemia affects renal sodium metabolism. In particular, we evaluated the production of nitric oxide (NO) and atrial natriuretic peptide – two mediators elevating cyclic guanosine monophosphate (cGMP) level and involved in the regulation of natriuresis and Na^+, K^+-ATPase activity.

MATERIALS and METHODS

Animals and reagents

The study was performed on 24 adult male Wistar rats weighing 237 ± 8 g (mean ± SEM) before the experiment. The animals were kept at a temperature of 20 ± 2°C with a 12-h light/dark cycle (lights on at 7.00 am). The study protocol was approved by the Bioethics Committee of the Medical University in Lublin.

The following reagents were obtained from Sigma-Aldrich (St. Louis MO, USA): bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX), EDTA, EGTA, Tris, Na_2ATP, TrisATP, ouabain and choline chloride. The specific inhibitor of all H^+, K^+-ATPases, 2-methyl-8-(phenylmethoxy)imidazol(1,2-α)pyridine-3-acetonitrile (Sch 28080) was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ, USA). Recombinant human leptin was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). As recommended by the manufacturer, the vial containing 5 mg of leptin was dissolved in 2.5 ml of 15 mM HCl and then 1.5 ml of 7.5 mM NaOH was added to bring pH to 5.2. This solution was diluted with the 15 mM HCl/7.5 mM NaOH mixture (5:3 v/v), frozen in 0.06 mg/0.25 ml aliquots, stored at –40°C and thawed immediately before use. BSA was dissolved in the same HCl/NaOH mixture and administered into control and pair-fed animals as a vehicle. Other reagents were of the highest analytical grade available.

Experimental protocol

The rats were randomized into 3 groups (n = 8 each): 1) control group, fed *ad libitum* standard rat chow containing 0.22% sodium and 0.5% potassium, 2) leptin-treated group, which received leptin injections (0.25 mg/kg twice daily *sc* for 7 days), 3) pair-fed group, in which food intake was adjusted to the leptin group. Control and pair-fed animals received *sc* injections of 0.25 mg/kg of BSA every 12 h. Previous studies have demonstrated that this dose of leptin raises its plasma concentration to the level comparable to that observed in obesity [39]. Before starting the respective treatments, 24-h urine was col-

lected in all animals kept in individual metabolic cages. The second urine collection was performed during the last 24 h of treatment.

The animals were anesthetized with pentobarbital (50 mg/kg) 6 h after the last injection and systolic blood pressure and heart rate were measured with the tail-cuff method. Then, the abdominal cavity was opened and blood was withdrawn from the abdominal aorta, punctured distally to the renal arteries, into EDTA-containing tubes. Subsequently, the aorta was ligated proximally to the renal arteries and 5 ml of physiological saline was infused slowly to remove erythrocytes from the kidneys. The kidneys were excised, separated into cortex and medulla, and microsomal fraction was isolated as previously described [6]. The kidneys, plasma, and urine samples were stored at –80°C until analysis. For the measurement of cyclic nucleotides, IBMX was added to the samples (30 µl of 10 mM IBMX per 0.5 ml of sample) to prevent breakdown of cGMP and cAMP by phosphodiesterases.

Measurement of renal ATPases

ATPase activity was assayed by measuring the amount of inorganic phosphate (P_i) liberated from ATP during the incubation of the microsomal fraction [7]. In brief, for the measurement of Na^+, K^+-ATPase, 50 µg of microsomal protein was incubated for 15 min at 37°C in the assay medium containing: 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 40 mM Tris-HCl (pH 7.4), 0.2 mM Sch 28080, and 3 mM Na_2ATP. Sch 28080 was added to block ouabain-sensitive H^+, K^+-ATPase, which interferes with Na^+, K^+-ATPase assay [7]. Then, P_i was assayed as previously described [7]. Na^+, K^+-ATPase activity (ouabain-sensitive fraction) was calculated as the difference between total ATPase (assayed in the absence of ouabain) and ouabain-resistant fraction, assayed in the presence of 2 mM ouabain, and was expressed in moles of P_i liberated by 1 mg of microsomal protein during 1 h (mol/h per mg of protein). Each sample was assayed in triplicate and the difference between averaged total ATPase and averaged ouabain-resistant ATPase was used in further calculations. Protein concentration in the microsomal fraction was assayed by the method of Lowry et al. [30].

For the ouabain-sensitive H^+, K^+-ATPase assay, 50 µg of microsomal protein was added to the incubation mixture containing 5 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris-HCl (pH 7.4) and 5 mM Tris-ATP. After 30 min, P_i was assayed as described above. The activity of ouabain-sensitive H^+, K^+-ATPase was calculated as the difference between the activities assayed in the absence and in the presence of 1 mM ouabain [7].

Kinetic analysis of renal ATPases

To get more insight into the function of renal ATPases, we measured their activities not only under optimal conditions but also at varying concentrations of activating ions and specific inhibitors. The relationship between Na^+ concentration and Na^+, K^+-ATPase activity was assessed by measuring enzyme activity in the presence of different sodium concentrations (10, 20, 30, 40, 60 and 100 mM, including Na^+ derived from ATP salt). When Na^+ concentration was varied, choline chloride was added at appropriate concentrations to maintain constant osmolality. The data were then analyzed using a cooperative model based on the Hill equation:

$$v = V_{max} \times [Na^+]^n/([Na^+]^n + K_{0.5}{}^n),$$

where: v is the actual enzyme activity, V_{max} is the maximal activity at saturating sodium concentration, $[Na^+]$ is actual sodium concentration, $K_{0.5}$ is a dissociation constant for Na^+ measuring the affinity of Na^+, K^+-ATPase for sodium and n is the Hill coefficient. In this model V_{max}, $K_{0.5}$ and n were allowed to vary to obtain the best fit for the data.

The effect of potassium concentration on Na^+, K^+-ATPase and ouabain-sensitive H^+, K^+-ATPase activities was fitted to the following equation:

$$v = V_{max} \times [K^+]/([K^+] + K_{0.5}),$$

where v is the activity measured at a given potassium concentration $[K^+]$, V_{max} is the maximal activity and $K_{0.5}$ is the dissociation constant for K^+. In Na^+, K^+-ATPase assay, 0.5, 1, 2, 5, 10 or 20 mM KCl was used, supplemented to 20 mM by choline chloride. In H^+, K^+-ATPase assay, K^+ concentration was established at 0.1, 0.2, 0.5, 1, 2 or 5 mM, supplemented to 5 mM by choline chloride.

The inhibition of Na^+, K^+-ATPase and H^+, K^+-ATPase by ouabain and of H^+, K^+-ATPase by Sch 28080 was analyzed according to the following formula:

$$v = V_{max}/(1 + [I]/[K_i]),$$

where v is the activity measured at a given concentration of inhibitor [I], V_{max} is the maximal activity

and K_i is the inhibition constant. Ouabain concentration varied from 10^{-7} to 10^{-3} M and Sch 28080 from 10^{-8} to 10^{-4} M in one-order steps [7]. The kinetic parameters were calculated by nonlinear least square regression separately for each animal and the values reported in Table 3 are means ± SEM from 5 samples in each group.

Renal function

Plasma and urinary creatinine was measured by the colorimetric method using Sigma-Aldrich kit (St. Louis, MO, USA). Plasma and urinary sodium and potassium were measured by flame photometry. Because phosphate is reabsorbed exclusively by the proximal tubule, we have measured phosphate excretion to evaluate whether leptin treatment affects transport in this segment. Plasma and urinary P_i was assayed by the method of Hurst [25]. For the measurement of phosphate and NO metabolites, plasma was deproteinized by filtration through 10 000 molecular weight cut-off filters (Ultrafree 0.5, Milipore, Bedford, MA, USA) upon centrifugation at 10 000 × g for 10 min.

NO metabolites, cyclic nucleotides, atrial natriuretic peptide and plasma leptin

NO metabolites (nitrates + nitrites, NO_x) were assayed in plasma and urine by the colorimetric method of Griess after enzymatic conversion of nitrates to nitrites by nitrate reductase, using Total Nitric Oxide Assay Kit (R&D Systems Ltd., Abingdon, Oxon, UK). Cyclic GMP and cyclic AMP were measured by competitive EIA using Cyclic GMP Enzyme Immunoassay Kit and Cyclic AMP Enzyme Immunoassay Kit, respectively (Cayman Chemical, Ann Arbor, MI). Atrial natriuretic peptide (ANP) was measured in plasma and urine using the rat ANP EIA kit (SPIbio, Massy, France). These methods were described in detail in our recent paper [7]. Plasma leptin was assayed using Leptin Enzyme Immunoassay Kit (Cayman Chemical).

Statistics

Data are presented as a mean ± SEM from 8 animals in each group. Pre- and post-treatment urinary excretion of creatinine, electrolytes, NO_x, ANP and cyclic nucleotides in a given group was compared by Student's *t*-test for related variables. Between-group comparisons were performed by ANOVA with Bonferroni's correction; $p < 0.05$ was considered significant.

RESULTS

The effect of leptin on food intake, water intake, body weight, plasma leptin, and blood pressure

There were no significant differences in food intake, water intake, and body weight between groups before treatment. Administration of vehicle had no effect on these variables in the control animals. Leptin tended to reduce food intake, but the difference became significant only on 6th and 7th day of treatment. Water intake and body weight did not differ between groups throughout the experiment. Plasma leptin concentration was by 208.8% higher in leptin-treated group. In contrast, pair-feeding induced 51.5% decrease in plasma leptin,

Table 1. The effect of leptin on food intake, water intake, body weight and plasma leptin concentration

	control	leptin-treated	pair-fed
food intake (g/day)†	31 ± 1	24 ± 1**	24 ± 2**
water intake (ml/day)†	26 ± 2	21 ± 3	25 ± 2
body weight (g)‡	230 ± 5	247 ± 9	244 ± 8
plasma leptin (ng/ml)	3.98 ± 0.15	12.26 ± 0.82***	1.93 ± 0.15*###

† during 7th day of treatment, ‡ after 7 days of treatment, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control group, ### $p < 0.001$ *vs.* leptin-treated group

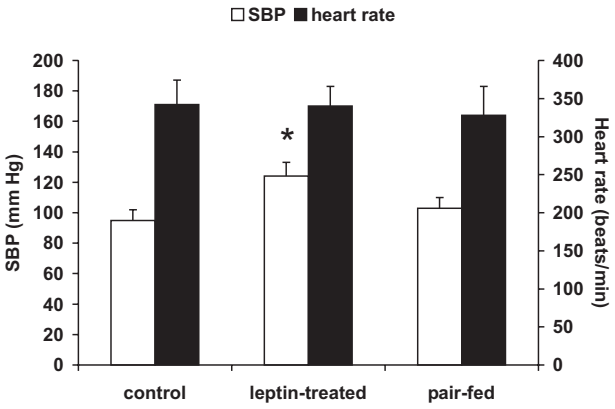

Fig. 1. The effect of leptin on systolic blood pressure (SBP, left axis) and heart rate (right axis). Leptin was administered *sc* at a dose of 0.25 mg/kg twice daily for 7 days. Control and pair-fed groups received bovine serum albumin at the same dose. In pair-fed group, food intake was limited to match the level observed in leptin-treated group. SBP and heart rate were measured 6 h after the last injection under general anesthesia. * $p < 0.05$, compared to control by ANOVA with Bonferroni's correction

possibly due to reduced food intake [39] (Tab. 1). Systolic blood pressure (SBP) was 30.5% higher in leptin-treated than in control group. In contrast, pair-feeding had no effect on SBP (Fig. 1). Heart rate was similar in all groups.

Urine output, urinary excretion of creatinine, electrolytes, NO_x, cyclic nucleotides and ANP was similar in all groups before treatment and did not change in the control group following vehicle administration (data not shown).

Renal function and electrolyte balance

Urine output and creatinine clearance were similar in all groups at the end of the experiment (Tab. 2). Plasma concentrations of sodium, potassium, phosphate and creatinine did not differ between groups. Due to reduced food consumption, sodium and potassium intake on 7th day of the experiment was by 22.0% lower in leptin-treated and in pair-fed group than in control animals. Daily urinary excretion of sodium was by 35.0% lower in leptin-treated than in control animals. In pair-fed group sodium excretion did not differ from control. There were no differences in K^+ excretion between groups. Leptin treatment had no effect on phosphate excretion. Absolute phosphate excretion tended to be lower in pair-fed group, but the difference did not reach the level of significance (Tab. 2).

Renal Na^+, K^+-ATPase and ouabain-sensitive H^+, K^+-ATPase

Na^+, K^+-ATPase activity in leptin-treated rats was by 32.4% higher in the renal cortex and by 84.2% higher in the renal medulla than in control animals (Fig. 2). In contrast, ouabain-sensitive H^+, K^+-ATPase activity was not affected by the leptin treatment (Fig. 3). Pair-feeding did not change the activity of either Na^+, K^+-ATPase or ouabain-sensitive H^+, K^+-ATPase.

Kinetic analysis of Na^+, K^+-ATPase revealed that leptin treatment increased V_{max} of the enzyme without affecting the affinity for sodium, the Hill coefficient, the affinity for potassium and K_i for ouabain. Leptin treatment resulted in an increase in V_{max} of Na^+, K^+-ATPase by 24.6% in the renal cortex and by 94.3% in the renal medulla (Tab. 3). Pair-feeding had no effect on these kinetic parameters in both regions of the kidney. Kinetic parameters of ouabain-sensitive H^+, K^+-ATPase (V_{max}, potassium affinity, K_i for ouabain and K_i for Sch 28080) did not differ between experimental groups (data not shown).

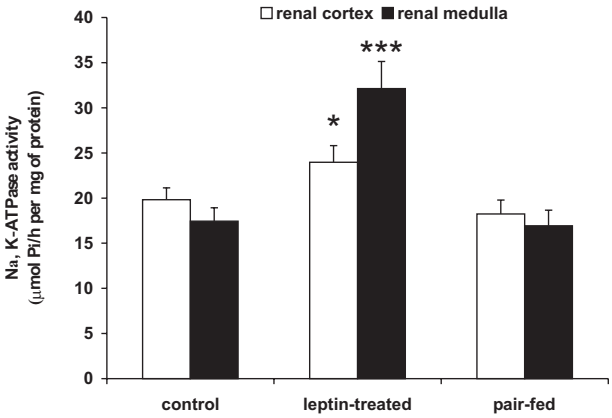

Fig. 2. Na^+, K^+-ATPase activity in the renal cortex and medulla in control, leptin-treated and pair-fed rats. Leptin was administered *sc* at a dose of 0.25 mg/kg twice daily for 7 days. Control and pair-fed groups received bovine serum albumin at the same dose. In pair-fed group, food intake was limited to match the level observed in leptin-treated group. * $p < 0.05$, *** $p < 0.001$, compared to control by ANOVA with Bonferroni's correction

NO metabolites, cyclic nucleotides and ANP

Plasma concentration of NO_x was similar in all groups. Urinary excretion of NO metabolites ($U_{NOx}V$) was by 55.0% lower in leptin-treated than in control group (Tab. 4). In pair-fed group, mean urinary NO_x excretion tended to be lower than in control group (–18.9%), but the difference did not reach the level of significance. $U_{NOx}V$ in pair-fed group was significantly higher (+80.3%, $p < 0.05$) than in leptin-treated group.

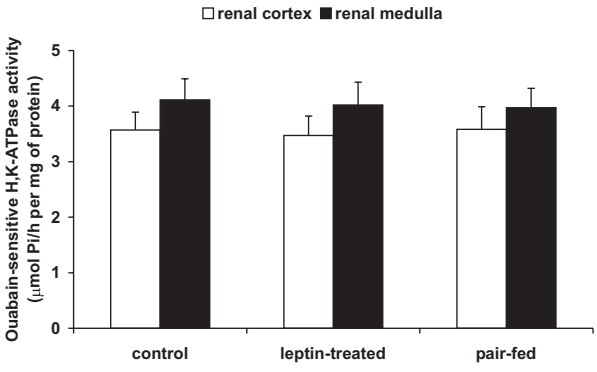

Fig. 3. Ouabain-sensitive H^+, K^+-ATPase activity in the renal cortex and medulla in control, leptin-treated and pair-fed rats. Leptin was administered *sc* at a dose of 0.25 mg/kg twice daily for 7 days. Control and pair-fed groups received bovine serum albumin at the same dose. In pair-fed group, food intake was limited to match the level observed in leptin-treated group

Table 2. The effect of leptin treatment on renal function and electrolyte balance

	control	leptin-treated	pair-fed
urine output (ml/day)	22 ± 4	18 ± 3	24 ± 4
creatinine clearance (ml/min)	2.23 ± 0.22	2.53 ± 0.24	2.14 ± 0.23
plasma Na^+ (mmol/l)	143 ± 3	138 ± 2	141 ± 2
plasma K^+ (mmol/l)	4.36 ± 0.25	4.21 ± 0.32	4.27 ± 0.37
plasma P_i (mmol/l)	2.34 ± 0.23	2.15 ± 0.19	2.80 ± 0.32
$U_{Na}V$ (mmol/day)	2.34 ± 0.21	1.52 ± 0.14*	2.24 ± 0.23$^{\#}$
Na^+ intake (mmol/day)†	2.95 ± 0.06	2.30 ± 0.1**	2.30 ± 0.2**
U_KV (mmol/day)	3.39 ± 0.57	3.20 ± 0.34	3.12 ± 0.32
K^+ intake (mmol/day)†	3.85 ± 0.08	3.00 ± 0.13**	3.00 ± 0.25**
$U_{Pi}V$ (mmol/day)	0.36 ± 0.04	0.33 ± 0.03	0.25 ± 0.03

$U_{Na}V$ – urinary excretion of sodium, U_KV – urinary excretion of potassium, P_i – inorganic phosphate, $U_{Pi}V$ – urinary excretion of inorganic phosphate; †during 7th day of treatment, * $p < 0.05$, ** $p < 0.01$ *vs*. control group, $^{\#}$ $p < 0.05$ *vs*. leptin-treated group

NO metabolites are filtered in glomeruli and then reabsorbed together with sodium by the renal tubules. Because leptin decreased urinary excretion of both NO_x and Na^+ without affecting creatinine clearance, it is possible that the decrease in $U_{NOx}V$ resulted exclusively from stimulation of tubular Na^+ and NO_x reabsorption. Therefore, we have calculated the $U_{NOx}V/U_{Na}V$ ratio in individual animals (Tab. 4). The amount of excreted NO_x per unit of excreted sodium was by 35.2% lower in leptin-treated than in control animals, but did not change significantly in pair-fed group. Thus, it is unlikely that lower $U_{NOx}V$ in animals receiving leptin resulted exclusively from enhanced tubular reabsorption.

Plasma level of cGMP in leptin-treated animals tended to be lower than in the control group and this difference was close to, but did not reach the level of significance ($p = 0.063$). Plasma cGMP in pair-fed group did not differ from control. Urinary excretion of cGMP ($U_{cGMP}V$) was by 26.3% lower in leptin-treated than in control group (Tab. 4). $U_{cGMP}V$ in

Table 3. Kinetic parameters of renal Na^+, K^+-ATPase

	Control	Leptin-treated	Pair-fed
		renal cortex	
V_{max} (μmol P_i/h per mg of protein)	18.6 ± 1.7	23.1 ± 2.1*	17.6 ± 1.8$^{\#}$
$K_{0.5}$ for Na^+ (mmol/l)	20.5 ± 1.9	21.1 ± 1.8	20.4 ± 0.2
n (Hill coefficient)	2.01 ± 0.20	1.96 ± 0.23	2.11 ± 0.21
$K_{0.5}$ for K^+ (mmol/l)	1.75 ± 0.09	1.68 ± 0.07	1.77 ± 0.11
K_i for ouabain (μmol/l)	121 ± 11	126 ± 8	118 ± 11
		renal medulla	
V_{max} (μmol P_i/h per mg of protein)	17.1 ± 1.8	33.3 ± 3.2***	16.5 ± 1.7$^{\#\#\#}$
$K_{0.5}$ for Na^+ (mmol/l)	23.2 ± 2.1	22.8 ± 2.1	23.4 ± 1.8
n (Hill coefficient)	2.04 ± 0.15	2.17 ± 0.21	2.11 ± 0.19
$K_{0.5}$ for K^+ (mmol/l)	1.86 ± 0.18	1.71 ± 0.19	1.77 ± 0.17
K_i for ouabain (μmol/l)	131 ± 9	127 ± 11	133 ± 8

V_{max} – maximal activity of Na^+, K^+-ATPase calculated in the analysis of the relationship between Na^+ concentration and enzyme activity, $K_{0.5}$ – dissociation constant, K_i – inhibition constant. * $p < 0.05$, *** $p < 0.001$ *vs.* control group, $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ *vs.* leptin-treated group

Table 4. The effect of leptin on plasma and urinary NO metabolites, cyclic nucleotides and ANP

	control	leptin-treated	pair-fed
plasma NO_x (μmol/l)	21.9 ± 2.4	19.7 ± 2.3	19.8 ± 1.9
$U_{NOx}V$ (μmol/day)	6.56 ± 0.78	2.95 ± 0.35***	5.32 ± 0.57$^{\#}$
$U_{NOx}V/U_{Na}V$ (μmol/mmol)	2.84 ± 0.24	1.84 ± 0.17*	2.48 ± 0.23$^{\#}$
plasma cGMP (nmol/l)	15.0 ± 1.7	10.3 ± 1.1	14.8 ± 1.6
$U_{cGMP}V$ (nmol/day)	75.6 ± 7.1	55.7 ± 5.5*	81.1 ± 8.1$^{\#\#}$
plasma cAMP (nmol/l)	55.0 ± 4.1	51.2 ± 3.2	57.6 ± 5.3
$U_{cAMP}V$ (nmol/day)	317 ± 37	301 ± 32	315 ± 28
plasma ANP (ng/l)	207 ± 21	262 ± 16	211 ± 18
$U_{ANP}V$ (ng/day)	2.42 ± 0.27	3.99 ± 0.34**	2.55 ± 0.18$^{\#\#}$

NO_x – nitric oxide metabolites (nitrites + nitrates), $U_{NOx}V$ – urinary excretion of NO metabolites, $U_{NOx}V/U_{Na}V$ – urinary NO_x excretion per unit of excreted sodium, $U_{cGMP}V$ – urinary excretion of cyclic GMP, $U_{cAMP}V$ – urinary excretion of cyclic AMP, $U_{ANP}V$ – urinary excretion of urodilatin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control group, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* leptin-treated group

pair-fed group was similar to control. In contrast to cGMP, plasma concentration and urinary excretion of cyclic AMP was similar in all groups (Tab. 4).

Plasma concentration of atrial natriuretic peptide tended to be higher in leptin-treated group but the difference was not significant. Urinary excretion of ANP increased in this group by 64.9%. Pair-feeding had no effect on either plasma or urinary ANP.

DISCUSSION

Na^+, K^+-ATPase, contained in basolateral membranes of renal tubular cells, is the driving force for tubular sodium reabsorption and thus is involved in the regulation of body fluid volume and blood pressure. Renal Na^+, K^+-ATPase activity is regulated by multiple hormones and neuromediators, and abnormalities of this regulation contribute to the development of arterial hypertension [18]. In contrast, ouabain-sensitive H^+, K^+-ATPase located in apical membranes of the tubular cells drives potassium reabsorption and urine acidification.

The results of this study demonstrate that 7-day treatment with leptin, at a dose which raises its plasma concentration to supraphysiological level comparable to that observed in obesity, induces arterial hypertension and decreases renal sodium excretion. Leptin had no effect on creatinine clearance, suggesting that decrease in natriuresis resulted from the stimulation of tubular Na^+ reabsorption. The effect on Na^+ transport may be accounted for, at least in part, by the stimulation of renal cortical, and especially medullary, Na^+, K^+-ATPase. In contrast to Na^+, K^+-ATPase, leptin did not affect ouabain-sensitive H^+, K^+-ATPase, consistently with lack of its effect on potassium excretion. Furthermore, no effect on phosphate excretion suggests that leptin stimulated Na^+ reabsorption outside the proximal tubule.

We found that leptin increased V_{max} of renal Na^+, K^+-ATPase without affecting other kinetic parameters. This may be accounted for by one of the following mechanisms [18]: 1) increased turnover rate of preexisting pump units, 2) redistribution of enzyme from inactive intracellular to active plasma membrane pool, 3) increased synthesis and/or impaired degradation of the enzyme. Further studies are required to discriminate between these possibilities.

One could suggest that leptin reduced natriuresis secondarily to decreased sodium intake. Several lines of evidence suggest that this was not the case. First, reduction of natriuresis was greater than reduction of Na^+ intake. Second, natriuresis did not change in pair-fed group in which sodium intake was identical as in leptin-treated group. Third, leptin had no effect on potassium excretion despite similar reduction of K^+ intake. These data suggest that Na^+ restriction was not of adequate magnitude and/or duration to trigger renal sodium conserving mechanisms.

The mechanism through which hyperleptinemia reduced natriuresis and increased Na^+, K^+-ATPase activity is not clear at present. Leptin activates sympathetic nervous system including renal sympathetic nerves [17, 23], which could result in activation of Na^+, K^+-ATPase and Na^+ transport [3, 16]. The re-

sults of this study suggest also an alternative explanation, i.e. deficiency of NO. Urinary NO_x excretion is used as an index of whole-body NO production [4]. Although $U_{NOx}V$ is affected also by alimentary intake of nitrates, it is unlikely that reduced $U_{NOx}V$ in leptin-treated rats was accounted for only by lower nitrate intake, because the decrease in $U_{NOx}V$ was larger (–55%) than the decrease in food intake (–22%), and the effect was not reproduced by pair-feeding. In addition, decreased excretion of cGMP confirms NO deficiency in leptin-treated rats. Measurement of urinary NO_x does not allow for identifying of the source of NO, however, it is likely that apart from systemic vasculature, the kidneys of leptin-treated animals generate less NO. One could suggest that reduction of $U_{NOx}V$ in leptin-treated rats results from the stimulation of Na^+ (and thus of NO_x) reabsorption. However, leptin treatment decreased the amount of excreted NO_x per unit of excreted sodium. Thus, increased tubular reabsorption could not entirely explain decreased urinary excretion of NO metabolites. NO produced by tubular and/or endothelial cells inhibits Na^+ reabsorption in most, if not all, nephron segments [35]. NO decreases Na^+, K^+-ATPase activity in proximal tubule [3, 29, 34, 44], medullary thick ascending limb [40] and medullary collecting duct [43]. In addition, chronic administration of NO synthase inhibitor increases renal Na^+, K^+-ATPase activity [27]. NO has been demonstrated to inhibit the transcription of α_1 subunit of Na^+, K^+-ATPase in cultured tubular cells [28], and another cGMP-activating mediator, uroguanylin, inhibits synthesis of γ subunit of the renal sodium pump [12]. Experimentally induced NO deficiency, especially in the renal medulla, leads to sodium retention and arterial hypertension [33] and reduced bioavailability of NO in the kidney is probably involved in the pathogenesis of hypertension in some rodent models such as Dahl salt-sensitive and spontaneously hypertensive rats [36]. Taken together, these data suggest that NO deficiency contributes to the up-regulation of Na^+, K^+-ATPase, reduced natriuresis, sodium retention, and arterial hypertension in leptin-treated rats.

The mechanism(s) leading to NO deficiency in leptin-treated rats cannot be identified in this study. In general, decreased NO generation results from lower expression and/or activity of nitric oxide synthase (NOS), reduced availability of substrate, L-arginine, or its impaired transport to NO-producing cells, deficiency of tetrahydrobiopterin (a cofactor of NOS), and excess of endogenous NOS inhibitors, such as asymmetric dimethylarginine. Furthermore, oxidative stress leads to accelerated breakdown of NO, which binds with superoxide to form peroxynitrite. Consequently, urinary NO_x excretion decreases because NO is sequestered in tissues rather than metabolized. Oxidative stress-related NO deficiency is involved in the pathogenesis of hypertension in many models, such as lead intoxication, uremic hypertension and angiotensin II-induced hypertension [36]. Leptin increases generation of reactive oxygen species in endothelial cells [11, 42], however, it remains to be established whether oxidative stress is responsible for leptin-induced NO deficiency.

Although not statistically significantly, plasma ANP was increased in leptin-treated animals. We suggest that this was the effect of hypertension since pressure overload up-regulates cardiac ANP synthesis. We also observed increased urinary excretion of ANP in leptin-treated rats. Because anti-ANP antibodies used by us demonstrate 100% cross-reactivity with nephrogenous natriuretic peptide, urodilatin, it is likely that we assayed urinary urodilatin rather than ANP itself. Although plasma ANP is filtered in glomeruli, it is almost completely metabolized by neutral endopeptidase contained in proximal tubules, and urinary excretion of true ANP is at least two orders of magnitude lower than detected by us [38]. Increased renal perfusion pressure stimulates renal generation of urodilatin [24], and was probably responsible for increased urinary "ANP" in our leptin-treated hypertensive rats. ANP and urodilatin are potent activators of receptor-bound guanylate cyclase and markedly increase plasma and urinary cGMP [41]. These peptides also enhance natriuresis and decrease renal medullary Na^+, K^+-ATPase activity [5]. Decreased excretion of cGMP and sodium despite stimulation of natriuretic peptide system suggests that hyperleptinemia induces resistance to natriuretic peptides, thus, leptin may be involved in the pathogenesis of the resistance to ANP observed in obesity [15].

The important limitation of the present study is that we measured blood pressure and heart rate under general anesthesia, which could reduce these parameters. However, it was not the main scope of this study to examine the effect of leptin on blood pressure. Hypertensive effect of adipose tissue hor-

mone has been described earlier [37], and our results only confirm those findings. In addition, cardiovascular parameters were measured under anesthesia also in the control group. In the subsequent studies, we evaluated these parameters in conscious animals and observed that leptin had the similar effect (unpublished observation).

Acute leptin administration stimulates natriuresis and endothelial NO generation [8, 9, 19, 26]. Therefore, blood pressure does not increase despite activation of the sympathetic nervous system. However, these effects are observed following high doses of this hormone and are short-lasting. Herein, we demonstrate that in contrast to acute, chronic hyperleptinemia causes NO depletion and antinatriuresis, the latter associated with the up-regulation of renal Na^+, K^+-ATPase. Renal sodium retention as well as vasoconstriction due to NO deficiency may contribute to leptin-induced hypertension as well as to the development of hypertension in hyperleptinemic obese subjects.

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REFERENCES

1. Agata J, Masuda A, Takada M, Higashiura K, Murakami H, Miyazaki Y, Shimamoto K: High plasma immunoreactive leptin level in essential hypertension. Am J Hypertens, 1997, 10, 1171–1174
2. Aizawa-Abe M, Ogawa Y, Masuzaki H, Ebihara K, Satoh N, Iwai H, Matsuoka N et al.: Pathophysiological role of leptin in obesity-related hypertension. J Clin Invest, 2000, 105, 1243–1252.
3. Aperia A, Holtback U, Syren ML, Svensson LB, Fryckstedt J, Greengard P: Activation/deactivation of renal Na^+, K^+-ATPase: a final common pathway for regulation of natriuresis. FASEB J, 1994, 8, 436–439.
4. Baylis C, Vallance P: Measurement of nitrite and nitrate levels in plasma and urine – what does this measure tell us about the activity of the endogenous nitric oxide system? Curr Opin Nephrol Hypertens, 1998, 7, 59–62.
5. Bełtowski J, Górny D, Marciniak A: The mechanism of Na^+, K^+-ATPase inhibition by atrial natriuretic factor in rat renal medulla. J Physiol Pharmacol, 1998, 49, 271–283.
6. Bełtowski J, Marciniak A, Wójcicka G, Górny D: The opposite effects of cyclic AMP-protein kinase A signal transduction pathway on renal cortical and medullary Na^+, K^+-ATPase activity. J Physiol Pharmacol, 2002, 53, 210–231.
7. Bełtowski J, Wójcicka G: Spectrophotometric method for the determination of renal ouabain-sensitive H^+, K^+-ATPase activity. Acta Biochim Pol, 2002, 49, 515–527.
8. Bełtowski J, Wójcicka G, Borkowska E: Human leptin stimulates systemic nitric oxide production in the rat. Obes Res, 2002, 10, 939–946.
9. Bełtowski J, Wójcicka G, Górny D, Marciniak A: Human leptin administered intraperitoneally stimulates natriuresis and decreases renal medullary Na^+, K^+-ATPase activity in the rat-impaired effect in dietary-induced obesity. Med Sci Monit, 2002, 8, BR221–BR229.
10. Bernal-Mizrachi C, Weng S, Li B, Nolte LA, Feng C, Coleman T, Holloszy JO et al.: Respiratory uncoupling lowers blood pressure through a leptin-dependent mechanism in genetically obese mice. Arterioscler Thromb Vasc Biol, 2002, 22, 961–968.
11. Bouloumie A, Marumo T, Lafontan M, Busse R: Leptin induces oxidative stress in human endothelial cells. FASEB J, 1999, 13, 1231–1238.
12. Carrithers SL, Hill MJ, Johnson BR, O'Hara SM, Jackson BA, Ott CE, Lorenz J et al.: Renal effects of uroguanylin and guanylin *in vivo*. Braz J Med Biol Res, 1999, 32, 1337–1344.
13. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP et al.: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med, 1996, 334, 292–295.
14. Correia ML, Haynes WG, Rahmouni K, Morgan DA, Sivitz WI, Mark AL: The concept of selective leptin resistance: evidence from agouti yellow obese mice. Diabetes, 2002, 51, 439–442.
15. Dessi-Fulgheri P, Sarzani R, Rappelli A: The natriuretic peptide system in obesity-related hypertension: new pathophysiological aspects. J Nephrol, 1998, 11, 296–299.
16. DiBona GF, Kopp UC: Neural control of renal function. Physiol Rev, 1997, 77, 75–197.
17. Dunbar JC, Hu Y, Lu H: Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. Diabetes, 1997, 46, 2040–2043
18. Feraille E, Doucet A: Sodium-potassium-adenosine-triphosphatase-dependent sodium transport in the kidney: hormonal control. Physiol Rev, 2001, 81, 345–418.
19. Fruhbeck G: Pivotal role of nitric oxide in the control of blood pressure after leptin administration. Diabetes, 1999, 48, 903–908.
20. Hall JE: Mechanisms of abnormal renal sodium handling in obesity hypertension. Am J Hypertens, 1997, 10, 49S–55S.
21. Hall JE, Brands MW, Henegar JR: Mechanisms of hypertension and kidney disease in obesity. Ann NY Acad Sci, 1999, 892, 91–107.
22. Hall JE, Louis K: Dahl Memorial Lecture. Renal and cardiovascular mechanisms of hypertension in obesity. Hypertension, 1994, 23, 381–394.

23. Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI: Receptor-mediated regional sympathetic nerve activation by leptin. J Clin Invest, 1997, 100, 270–278.
24. Heringlake M, Wagner K, Schumacher J, Pagel H: Urinary excretion of urodilatin is increased during pressure natriuresis in the isolated perfused rat kidney. Am J Physiol, 1999, 277, F347–F351.
25. Hurst RO: The determination of nucleotide phosphorus with a stannous chloride–hydrazine sulphate reagent. Can J Biochem, 1964, 42, 287–292.
26. Jackson EK, Li P: Human leptin has natriuretic activity in the rat. Am J Physiol, 1997, 272, F333–F338.
27. Kang DG, Kim JW, Lee J: Effects of nitric oxide synthesis inhibition on the Na, K-ATPase activity in the kidney. Pharmacol Res, 2000, 41, 121–125.
28. Kone BC, Higham S: Nitric oxide inhibits transcription of the Na^+-K^+-ATPase alpha1-subunit gene in an MTAL cell line. Am J Physiol, 1999, 276, F614–F621.
29. Linas SL, Repine JE: Endothelial cells regulate proximal tubule epithelial cell sodium transport. Kidney Int, 1999, 55, 1251–1258.
30. Lowry OH, Rosenbrough NI, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem, 1951, 193, 265–275.
31. Margetic S, Gazzola C, Pegg GG, Hill RA: Leptin: a review of its peripheral actions and interactions. Int J Obes Relat Metab Disord, 2002, 26, 1407–1433.
32. Mark AL, Shaffer RA, Correia ML, Morgan DA, Sigmund CD, Haynes WG: Contrasting blood pressure effects of obesity in leptin-deficient ob/ob mice and agouti yellow obese mice. J Hypertens, 1999, 17, 1949–1953.
33. Mattson DL, Lu S, Nakanishi K, Papanek PE, Cowley AW Jr: Effect of chronic renal medullary nitric oxide inhibition on blood pressure. Am J Physiol, 1994, 266, H1918-H1926.
34. McKee M, Scavone C, Nathanson JA: Nitric oxide, cGMP, and hormone regulation of active sodium transport. Proc Natl Acad Sci USA, 1994, 91, 12056–12060.
35. Ortiz PA, Garvin JL: Role of nitric oxide in the regulation of nephron transport. Am J Physiol Renal Physiol, 2002, 282, F777–F784.
36. Pallone TL, Mattson DL: Role of nitric oxide in regulation of the renal medulla in normal and hypertensive kidneys. Curr Opin Nephrol Hypertens, 2002, 11, 93–98.
37. Shek EW, Brands MW, Hall JE: Chronic leptin infusion increases arterial pressure. Hypertension, 1998, 31, 409–414.
38. Shin SJ, Wen JD, Chen I H, Lai FJ, Hsieh MC, Hsieh TJ, Tan MS et al.: Increased renal ANP synthesis, but decreased or unchanged cardiac ANP synthesis in water-deprived and salt-restricted rats. Kidney Int, 1998, 54, 1617–1625.
39. Steinberg GR, Bonen A, Dyck DJ: Fatty acid oxidation and triacylglycerol hydrolysis are enhanced after chronic leptin treatment in rats. Am J Physiol, 2002, 282, E593–E600.
40. Syren ML, Tirelli AS, Assael BM, Sereni F: Regulation of sodium-potassium-adenosine triphosphatase activity by extracellular guanosine 3',5'-cyclic monophosphate in rat kidney Acta Physiol Scand, 1996, 158, 295–296
41. Wong KR, Xie MH, Shi LB, Liu FY, Huang CL, Gardner DG, Cogan MG: Urinary cGMP as biological marker of the renal activity of atrial natriuretic factor. Am J Physiol, 1988, 255, F1220–F1224.
42. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M: Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. J Biol Chem, 2001, 276, 25096–25100.
43. Zeidel ML, Silva P, Brenner BM, Seifter JL: Cyclic GMP mediates effects of atrial peptides on medullary collecting duct cells. Am J Physiol, 1987, 252, F551–F559.
44. Zhang C, Mayeux PR: NO/cGMP signaling modulates regulation of Na^+, K^+-ATPase activity by angiotensin II in rat proximal tubules. Am J Physiol, 2001, 280, F474–F479.
45. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. Nature, 1994, 372, 425–432.

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